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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/21/74

JAN 29 1974

1. Principal Investigator (give title and degrees):
Louis A. Soloff, M.D.
Blanche P. Levy, Distinguished Service Professor of the University; Professor of Medicine; Director, Research Lipid Laboratory; Member, Research Advisory and Review Committee for the Clinical Research Center.

2. Institution & address:
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3401 North Broad Street
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3. Department(s) where research will be done or collaboration provided:
Research Lipid Laboratory
Division of Cardiology
Department of Medicine
Temple University Health Sciences Center

4. Short title of study:
Role of lecithin:cholesterol acyltransferase (LCAT) in cholesterol metabolism in health, disease and during smoking.

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5. Proposed starting date: July 1, 1974

6. Estimated time to complete: Three years.

7. Brief description of specific research aims:

LCAT is an essential factor in plasma cholesterol esterification. For this reason, a study of this enzyme should increase our overall knowledge of lipid metabolism. There is already evidence that patients with LCAT deficiency develop atherosclerosis even though their levels of blood cholesterol and beta-lipoproteins are far below normal. It therefore appears worthwhile to determine whether LCAT plays a role in other patients with atherosclerosis and if so, possibly to add another method of separating genetic from environmental factors. So called risk factors, including smoking and resistance to these risk factors cannot be evaluated if genetic factors are ignored. Purified LCAT appears essential to supply more complete information on the role of LCAT in lipid metabolism in health and disease. We already have made a preparation of human LCAT which is stable and 4000 to 10,000 times as concentrated as that present in human plasma. We aim 1) to produce LCAT antibodies for isolation and quantification of pure LCAT 2) to determine LCAT's physical, chemical and enzymatic properties 3) to determine LCAT activity during physical, pharmacologic and hormonal stimuli and during nicotine administration and smoking in the experimental animal, in an invitro system containing plasma with and without LCAT and red blood cells, and arterial wall red blood cell plasma with and without LCAT and in humans with and without obstructive coronary heart disease documented by coronary arteriography

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8. Brief statement of working hypothesis:

Glomset[1] postulated that plasma lipoproteins pick up cholesterol from a variety of cells by a mechanism involving equilibrium between lipoprotein bound and cell free cholesterol. LCAT is postulated to convert free cholesterol to cholesterol esters which are removed from plasma, hydrolyzed by the liver and transformed into bile. As a result of plasma esterification, the equilibrium may be shifted so as to allow egress of cholesterol from cells. More recently, Glomset and Norum[2] have postulated an 180° opposite mechanism, namely, that LCAT functions to maintain a normal content of cell membrane cholesterol. In either event the result might be an alteration in cell surface which prediposes it to the accumulation of cholesterol. That LCAT might play a role in atherosclerosis is suggested by 1) our findings that LCAT activity is inversely related to species susceptibility to atherosclerosis (manuscript[3]), 2) that an atherogenic diet inhibits cholesterol esterification[4], 3) our findings that the fractional rate of cholesterol esterification of cholesterol is abnormal in familial hyperbetalipoproteinemia[5], 4) Gjone's demonstration of severe atherosclerosis at autopsy in a 40 year old woman who died with LCAT deficiency and a blood cholesterol of only 60mg/dl. and his findings of atherosclerosis in other patients with LCAT deficiency. He writes "Why these patients with this lack of plasma cholesterol esters and low levels of apolipoprotein B develop atherosclerosis remains to be explained"[7], and 5) evidence that high density lipoprotein apoprotein, poor in surface cholesterol and with a high sphingomyelin/lecithin ration, postulated effects of LCAT, is most capable of removing membrane cholesterol[8]. Smoking LCAT interrelations will be studied, particularly in atherosclerotic patients.

9. Details of experimental design and procedures:

We intend to seek answers to these problems in several different ways based upon our past work on LCAT and on work done by others. A summary of our work on LCAT was presented at a private international symposium on LCAT in October, 1973 in Oslo, Norway. This symposium including our manuscript[9], a copy of which is inclosed, will be published in April, 1974 as a supplement to the Scandinavian Journal of Laboratory and Clinical Investigation. So far as we know, we are the only ones who has prepared a stable preparation of LCAT which is 4,000 to 10,000 times the concentration present in normal human plasma.

At the present time we are testing the activity of this preparation in an invitro system containing apolipoprotein glutamine I and II, the putative natural activator and inhibitor of this enzyme.

However, there are other possible activators and inhibitors as shown in our work with heparin[10] (manuscript enclosed accepted for publication by Biochimica et Biophysica Acta). The electron morphologic changes that occur in such studies are being studied by Dr. Hisashi Shinozuka, Associate Professor of Pathology and member and electronmicropathologist, Fels Research Institute of our Medical School. These electronmicroscopic observations will test Shumaker and Adams's hypothesis that LCAT has as one of its functions the maintenance of lipoprotein structure as triglycerides are removed from very low density lipoproteins.

However, because of the possibility of other as yet unknown inhibitors and activators of LCAT activity, because of the small amount of the enzyme present in the plasma and also because of the complex interaction between the enzyme and its substrates we think it is imperative to develop a method that assures purity of the enzyme and is capable of measuring accurately minute amount of the enzyme in the range found in the plasma and of the very minute changes that might occur in different states. For this reason, we have chosen an immunologic approach in an attempt to solve this problem.

(see following pages)

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Radioimmuno assay of LCAT

Methods of procedure:

1. Production and characterization of antibody to LCAT

a) Purification of antigen (LCAT):

Our laboratory is one of two in which highly purified LCAT has been obtained. We have worked out a procedure for preparing greater than 4,000 fold purified LCAT which is stable for at least eight hours, with a yield of 5%.

A litre of outdated plasma is first mixed with 20 ml of 10% egg lecithin suspension and 1 ml of mercaptoethanol and 440 gms of amonium sulfate. The precipitate after collection by centrifugation is dissolved in 0.005 EDTA pH 7.4 and its density is adjusted to 1.22 with cesium chloride. The LCAT is layered under Cscl of d=1.21 and floated by centrifugation at 100,000 g. for 36 hours. LCAT bands at a density of 1.20 where most of the lipoproteins float at d=1.18 and most of the plasma proteins settle at the bottom at 1.26. The clear zone between the plasma proteins and lipoproteins is dialysed and fractionated on a DE-52 column using tris-EDTA-Nacl pH 7.4 and a sodium chloride gradient. The eluted enzyme is then put on a HDL-sepharose column and washed with tris-EDTA-Nacl buffer. After the protein peak has come out the enzyme is eluted with 0.05 tris-0.4M glycine buffer pH 9.0. The protein was estimated in all these steps by the fluorscamine method[11] after dialysis. About 500 ug of enzyme is obtained with a 5% yield. On standard polyacrylamide gel electrophoresis two bands are seen and the enzyme activity is associated closely with the fast moving band.

On immunoelectrophoresis with hyperimmune equine serum produced against whole human serum (copy of photograph enclosed) a single arc is the only one obtained. We are currently affinity labelling the enzyme with radioactive diisopropyl fluoro-phosphate to identify the enzyme bands. Since those obtained on immunoelectro-phoresis are close to that of HDL and albumin the possibility that albumin and/or HDL is contaminating the enzyme exists. These are currently under study.

b) Production of antibody.

100 ug of purified LCAT will be administered into young rabbits intravenously in 0.1 to 0.2 ml of saline. On the fifth day the animals would receive 100 ug of the enzyme with completc adjuvant (1:L) intramuscularly. Weekly intramuscular injections of 100 ug will be given thereafter. The animals will be bled weekly and their antibody production will be checked by crossed immunoelectrophoresis[12]. Once high antibody titer is obtained by immunoelectrophoresis (a method of higher resolving power) the enzyme and antisera will be carried out to check for the presence of possible contaminants in the enzyme preparation and their antibodies in antisera.

c) Characterization of antibody.

If contaminating antibodies are found in the antisera, they will be first identified by using pure plasma proteins (albumin, HDL etc) and then absorbed out. In order to purify and enrich LCAT antibody, the antiserum, after removing the contaminating antibody with the pure contaminating antigen will be put on a sepharose-LCAT column. After eluting non antibody proteins the LCAT antibody will be eluted with a gradient of quanidine-Hcl as suggested by Weintraub and Kadesky[13]. The fractions will be

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assayed by radioimmuno assay (see below) and those showing the highest titre will be used as purified antibody.

For purposes of establishing absolute identity of anti LCAT antibody, $DFPH^3$ labelled LCAT will be used as antigen and precipitates obtained by the double antibody technique of Weintraube and Rose[14]. The formation of precipitates containing H^3 label will be considered as indicative of the presence of antibody to LCAT. For the further characterization electrophoretic and chromatographic techniques using $DFPH^3$-LCAT incubated with antiserum will be used[15].

Radioimmuno assay: The highly purified preparation obtained after affinity chromatography will be concentrated by vacumn dialysis to give .5 to 1 mg/ml enzyme solution. After adjusting pH to 7.6 with 1.0 M monosodium phosphate the sample will be injected into bottles containing carrier free I^{125} of 1-5 millicurie. 216.0 mg. of chloramine T and 0.3 mg of potassium iodide will be added and the reaction mixture will be incubated for 30 minutes at room temperature. The reaction will be stopped by adding sodium bisulfite. The unreacted iodine will be removed by sephadex chromatography using G-25. A protocol for the assay is given:

Unlabelled enzyme or serum	0.5 ml *
Labelled enzyme diluted to contain approximately 2-10 ug	0.1 ml *
Antibody - diluted rabbit antiserum	0.1
First incubation	1-5 days
Antibody to rabbit globulin	0.1 ml
Incubation	48 hrs.

* unlabelled:labelled to vary from 0.9 to 0.1 (w/w)

The assay will be performed in equivalent zone and the condition of the assay system like the dilution of LCAT antiserum, the antiserum to rabbit γ globulin, period of incubation etc will be determined using the standard of known protein concentration and known iodine labelled enzyme. The precipitated I^{125} after 2 nd incubation will be collected by centrifugation and the counts in the precipitate and supernatant will be determined. The dose response curve will be constructed after logit transformation as suggested by Rodbard[16] and the following statistics will be evaluated on a sequential and cummulative basis using wang-300 programmable calculator.

a) % radioactivity bound in the absence of unlabelled enzyme %B
b) Slope of the linear position of %B vs log dose curve
c) 50% intercept
d) within assay standard deviation
e) between assay standard deviation

Effect of physicochemical factors on the assay:

Since the enzyme has been proposed to exist as a complex with HDL[17] the effect of pH 9.0 and taurocholate, agents known to dissociate LCAT from HDL, will be studied on the radioimmuno assay. At pH 9.0 the antigen-antibody complex formation will not be expected to be seriously affected. The effect of agents that inhibit LCAT such as sulfhydryl blockers, reagents, polyvalent cations will also be studied on the antigen antibody reaction.

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Application to Plasma:

From the yield of purified enzyme obtained by Fielding and Fielding[18] and in our laboratory the concentration of LCAT in plasma may be calculated to be approximately 0.1-0.5 mg%. Hence each assay system will contain about 0.1 ug of labeled enzyme in 0.1 ml and plasma diluted to give approximately 0.01 to 0.9 ug LCAT (two times to 100 times). After carrying out the incubation as mentioned in the protocol the labell in the precipitate and supernatant will be determined. It should be possible to carry out about 100 samples with each labelled batch of enzyme. The purified enzyme will be used as a standard, defined arbitrarily as 1 unit/ug of protein. For studies on pathological serum 6-10 normals will be used as separate controls. The values obtained will be compared with the enzyme activity determined by GLC.

Animal Studies:

Since the ultimate goal of the research project is to evaluate the role of LCAT in the homeostasis of plasma cholesterol and in atherosclerosis, we plan to study the effect of LCAT infusion in experimental animals and its effect on plasma cholesterol metabolism. Rabbits and guinea pigs are selected for this purpose since the former has been extensively used as an experimental model for atherosclerosis and the latter has been shown to develop lipoprotein abnormalities similar to that in LCAT deficient humans, when these animals are fed cholesterol. However repeated infusions of LCAT may produce immunological reactions. These will be circumvented by the use of antilymphocyte serum.

Experiments with rabbits:

A group of 20 rabbits will be divided into groups of eight and 12 and the latter will be fed cholesterol containing diet for 20 days. These will be divided into three groups 1) four animals left untreated 2) four animals treated with antilymphocyte serum (ALS) 3) four animals treated with ALS and LCAT. Those on normal diet will be divided into two groups of four and one will be treated with ALS. Concentrated purified LCAT will be administured intravenously every third day and ALS will be administered once a week. Before and after the experimental period (15 days) the following will be compared in all five groups: cholesterol levels of plasma (free and ester) and presence geographically of atherosclerotic lesions and if necessary their lipid composition.

Guinea Pigs:

Similar experiments will be carried out in guinea pigs but in addition to determing serum cholesterol levels and the presence of atherosclerosis, the lipoproteins will be analyzed for abnormal LDL and HDL using gel exclusion chromatography and electron microscopy. The effect of LCAT treatment on plasma cholesterol levels in these animals, on atherosclerotic plaques in the rabbit and on the abnormal lipoproteins of guinea pigs will be determined.

While attempting to develop radio immuno assay of LCAT, we are evaluating two assay systems for their respective efficiency and applicability to the routine measurement of the initial rate of esterification of cholesterol in human serum. We are comparing our modification of the Stokke and Norum method with a GLC method introduced by Marcel et al[19]. Their values are lower than ours. Furthermore their method has a relatively large error (± 8%) and was performed on too few subjects to allow for firm conclusions on the relative merit of their technique.

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The role of erythrocytes in cholesterol metabolism:

Preliminary experiments in our laboratory have shown that erythrocyte cholesterol is probably esterified at a faster rate than plasma cholesterol.

TABLE 1

12 ml of fresh or heated serum (30 minutes at 56°C) were incubated with or without 3 ml. of erythrocytes. The amounts of free and total cholesterol were estimated at 0 and 6 hours.

Chol. mg.	Fresh Serum		Heated Serum		Fresh Serum & Erythrocytes		Heated Serum & Erythrocytes	
Plasma	0	6 hrs	0	6 hrs	0	6 hrs	0	6 hrs
Total	15.6	15.7	15.6	15.6	15.6	16.4	15.6	16.7
Free	4.8	3.4	4.8	4.6	4.8	3.4	4.8	4.7
Ester	10.8	12.3			10.8	13.0	10.8	12
ΔEster	1.5		0.2		2.2		1.2	
Erythrocytes								
Free	14				1.6	0.59	1.16	0.55
ΔFree					0.57		0.61	

The presence of erythrocyte in both cases (fresh and heat inactivated plasma) causes substantial increase in the extent of cholesterol esterification. This may be due to: 1) erythrocytes containing LCAT 2) Erythrocyte cholesterol is a better substrate than plasma.

It has been shown in the rat that erythrocytes have only 2% of plasma LCAT activity. Also, D'Hollander and Chevallier[20] injected rats with C^{14} cholesterol and studied the exchange of cholesterol between plasma and erythrocytes in relation to cholesterol esterification. They concluded that erythrocyte cholesterol was esterified before it mixed with the free cholesterol of plasma. They also observed the rate of esterification of cholesterol in blood to be much higher than that of plasma alone. We have analyzed the data of Hagerman and Gold[21] on the exchange of C^{14} cholesterol between plasma and erythrocytes in the case of dogs in vitro, and have computed the LCAT activity from their data using plasma and erythrocyte cholesterol as substrate (Table II). It is clear that if plasma cholesterol is the substrate the computed LCAT activity would be too high a value compared to experimentally obtained plasma LCAT activity.

TABLE II Rate of cholesterol esterification during exchange of plasma and erythrocytes when one of the compoment is labelled.

Assumed Substrate	Rate of Esterification/gm of blood/hr. Rat (a)	Dog (b)
Plasma	120 ug	180 ug
Erythrocyte	70 ug	40 ug
Observed rate other methods	50 ug (c)	40 ug (c)

(a) reference #20
(b) reference #21
(c) our laboratory estimation

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The following experiments are designed to confirm our preliminary findings in humans using more sensitive methods and to further elucidate the nature of the cholesterol pools of the erythrocyte. In view of the total and rapid exchange of cholesterol between plasma and erythrocyte in vivo and the lack of the same under in vitro conditions separate pools may exist in erhythrocytes of which only one may function as substrate for LCAT in vitro. We postulated that transit of erythrocytes through the capillaries causes a redistribution and homogenization of erythrocyte pools. Or, erythrocytes depleted of their cholesterol by the action of LCAT may regain their cholesterol compliment by physical contact with the membranes of blood vessels, plasma membranes of liver, kidney, etc. These will be approached by in vivo experiments using rat as an experimental animal.

Methods of Approach

Blood from fasting normal human subjects will be collected in EDTA coated tubes. For the determination of plasma free and total cholesterol 2 ml. of aliquots of blood will be chilled in ice and the erythrocytes will be separated by centrifugation at 1000g for 10 minutes. At 4°C the erythrocytes will be washed four times with saline (0.9% and pH 7.3). The washings will be pooled with plasma and lipids will be extracted with 40 volumes of chloroform-methanol (2:1). A known aliquot will be dried under N_2. The erythrocyte lipids will be extracted with 80 volumes 2:1 chloroform-isoproponol and an aliquot will be dried under N_2. Total and free cholesterol will be determined by gas liquid chromatography techniques[21] using automatic sample injector and electronic integration attachment. Five cholestane will be used as an internal standard. Changes in free cholesterol will be considered as equal to increase in esterified cholesterol.

For determination of specific radioactivity 10 to 20 ug of total cholesterol will be spotted on TLC plates and developed using petroleum either;ether:acetic acid (80:15:2) as solvent. The cholesterol and ester spots will be extracted with acetone. An aliquot will be used for estimation of amount of cholesterol by GLC and another for radioactivity.

For invitro radioactive labelling of plasma H^3 or C^{14}, cholesterol in acetone will be slowly added to 5% albumin to make an emulsion which will be added to plasma (0.1 V plasma) for in vivo experiments with rats' blood (cholesterol will be labelled) by injecting H^3 meralon and blood drawn by cardiac puncture. Administration of this labelled RBC will be done through the tail vein.

Approaches: 1) To determine the effect of erythrocyte on plasma LCAT activity blood and plasma will be incubated separately for 0.5, 1,2,4,8, and 24 hours. Free and total cholesterol of plasma and erythrocytes will be determined for each period. Change in free cholesterol would be considered as equal to the cholesterol ester formed. Blood would be expected to show more of the esterification compared to that of plasma. Also, the esterification loss of erythrocyte cholesterol would reach a plateau at about eight hours.

2) To find out whether erythrocyte cholesterol is a better substrate. C^{14} labelled plasma will be incubated for one hour at 37°C. At this time called 0 time red cells will be mixed to give a hematocrit of 40%. After incubation for 0.5, 1,2, and 12 hours at 37°C specific activity and amounts of free and total ester cholesterol will be determined. A control without added red cells will be carried out throughout the procedure. Data will be analyzed as follows.

Rate of esterification for time period 't' = $\frac{\text{change in total label in cholesterol ester for time period 't'}}{\text{average specific activity of cholesterol substrate for the time period}}$

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The rate of esterification computed using plasma and erythrocyte cholesterol average specific activities will be compared with the control plasma's rate. It is expected that if erythrocyte is really the substrate for LCAT, the ratio of esterification rate using specific activity of erythrocyte cholesterol to the control's rate of esterification will be closer to unity. When plasma specific activity is used to determine the rate of esterification in blood the ratio will be far less than 1, especially in the early period of incubation since the enxhcange has proceeded only very little and the specific activity of plasma cholesterol will be high (compared to that of red cells).

3) To test whether a specific pool cholesterol on red cells is the substrate for LCAT, exchange between plasma dn erythrocyte cholesterol has been reported to be completed in 8-12 hours in vitro. Such red cells take up cholesterol when incubated with heated plasma. Thus the cholesterol lost through LCAT action could be replenished by labelled H^3 cholesterol if red cells are incubated in the heated plasma labelled with H^3 cholesterol. The 12 hour incubated mixture of experiment 22 will therefore be reincubated in heated plasma labelled with H^3 cholesterol for six hours. Samples will be incubated using this dcubly labelled erythrocyte and unlabelled fresh plasma. The ratio of C^{14} to H^3 specific activity of cholesterol ester at different periods of incubation will be determined . This will be unity if there is only one pool for LCAT to act. On the other hand if LCAT substrate of erythrocyte was coming from a peparate pool which is relabelled with H^3, the ratio will be much less than unity. These experiments will be repeated with at least 10 different blood samples and in all cases student 't' tests will be performed when controls are compared with experimental samples. These three experiments will be repeated with rat blood. In addition plasma and red blood cells will be labelled in vivo by intravenously injecting them with C^{14} mevalonate. Experiments using labelled (in vivo) plasma and unlabelled rat red cells and vice versa along the lines used in experiments will be done. The calculations and analysis of data will be the same.

4) To determine whether factors in circulation are involved in the control of red cell cholesterol pools. Blood will be pooled from six rats and separated into plasma and cells. C^{14} labelled blood will be incubated at 37°C for six hours to deplete the cell cholsterol. These cells will be infused into six rats through the tail vein. After 30 minutes, blood will be collected and cells will be separated. C^{14} of these cells will be determined. An aliquot of the labelled cells which have been preincubated with plasma for six hours will be mixed with unlabelled red cells to give the same C^{14} counts per ml of packed cells as that found for infused cells. These two kinds (one mixed in vitro with unlabelled red cells and the other mixed invivo) of red cells will be incubated witn unlabelled plasma and the rate of cholesterol ester C^{14} formation will be compared. If factors in circulation affected LCAT substrate of red cell an increased efficiency (more C^{14} in ester) will be noted in the case of infused cells.

Once the role of RBC as a substrate for LCAT reaction has been established and quantified, we shall return to the study of the interaction of arterial wall, RBC and plasma with and without LCAT activity. Preliminary studies in our laboratory suggest that these interactions have an important effect on intravascular and vascular cholesterol metabolism.

<u>Interactions of human serum lipoproteins and LCAT with platelets:</u>

We have observed that LDL strongly potentiated ADP induced platelet aggregation

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in suspensions of washed human platelets. On the other hand VLDL inhibited platelet aggregation. LDL did not cause platelet aggregation in the absence of ADP.

These observations have stimulated the following studies primarily proposed by Stefan Niewiarowski, M.D., Ph.D., who is Research Professor of Medicine and member of the specialized sector for Thrombosis Research at our Medical School. The lipid studies will be done in our laboratory and the hematological studies will be done in Dr. Niewiarowski's laboratory.

a) Study on the effects of isolated serum lipoproteins on the aggregation and release reaction of human platelets; b) the interrelationship between plasma and platelet lipoproteins; c) effects of platelets and platelet components on the activity of lipoprotein lipase (LP) and lecithin-cholesterol acetyltransferase. These three parts of our project will be discussed separately.

a) Lipoprotein will be prepared by the method of Havel, et al[23] as modified by Hatch and Lees[24]. This method is based on the sequential flotation of lipoproteins by preparative ultracentrifugation. The lipoproteins are floated by increasing the density of each step by the addition of sodium bromide to the medium. The lipoproteins fractions are further purified by refloating them twice at the following densities: very low density lipoproteins (VLDL) at D=1.06; low density lipoproteins (LD) at 1.062; and high density lipoproteins (HDL) at 1.21. Further purification of lipoproteins if necessary will be achieved by gel filtration using sephadex G-200, sepharose or biogel columns. Since free fatty acids may cause platelet aggregation, the free fatty acid content of the purified lipoproteins will be determined by the method of Dole and Meinert[25]. Homogeneity of lipoproteins will be checked by electrohoresis and immunodiffusion. Platelets will be prepared from human blood, collected in blood bank on acid citrate dextrose anticoagulant, by means of the method Mustard, et al[26]. In this method apyrase is sued to prevent platelet aggregation during centrifugation. After washing, platelets are resuspended in Tyrode-albumin solution. These platelets are stable for at least three hours at 37°C, they are sensitive to low concentration of ADP, collagen and thrombin. Their ultrastructure and shape are preserved. ADP causes platelet aggregation in this suspension but it does not cause any significant release of platelet constituents, Thrombin and collagen cause both aggregation and release. Sensitivity of washed platelet to epinephrine is usually decreased. Washed platelets will be labeled with ^{3}H serotonin[27] for the purpose of study the platelet release reaction.

In typical experiments lipoprotein fractions will be incubated with the platelet suspensions before studying platelet aggregation induced by the four stimuli (ADP, collagen, thrombin, epinephrine) in the Payton aggregometer. After aggregation, platelets will be centrifuged in the Eppendorf centrifuge and the supernatant will be tested for the presence of the released platelet constituents such as ^{3}H serotonin, adenine nucleotides and platelet factor 4[24]. The increased availability of platelet factor 3 during platelet aggregation and the effect of lipoproteins on this reaction will be tested. Platelet factor 3 activity will be evaluated by means of Russels vipor venom following the procedure described by several investigators[28]. The effect of various inhibitors of platelet aggregation such as dipyridamole derivatives, aspirin, prostaglandin E_1 on the potentiation of platelet aggregation by lipoproteins will be studied.

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In another set of experiments, lipoproteins will be prepared from serum of patients with various types of hyperlipoproteinemia. The effect of these fractions on platelet aggregation and the platelet release reaction will be studied. Similarly, platelets will be prepared from the blood of patients with hyperlipoproteinemia and their sensitivity to various aggregating agents will be investigated.

Finally washed platelets will be suspended in normal plasma and in plasma of patients with abetalipoproteinemia. Aggregation of these platelets in the presence of increasing concentration of added LDL will be studied. Similar experiments will be performed using platelets isolated from the blood of patients with hyperlipoproteinemia and abetalipoproteinemia.

b) The interrelationship between plasma lipcproteins and platelet lipoproteins is not known. Studies in various laboratories are being carried out on the exchange between platelet and plasma lipids. We would like to determine whether platelet and plasma betalipoproteins have common antigens such as those belonging to the Lp or Ag system. The antisera will be prepared and antigen typing performed according the methods of Berg[29]. If platelets and plasma share common lipoprotein antigens, it is more likely that changes in the leve of plasma betalipoprotein will affect platelet function.

c) As it has been mentioned previously there are controversial data regarding the effect of platelets on lipoprotein lipase (LPL). Some authors describe platelets as containing an inhibitor of lipoprotein lipase[30,31] while Bucher[32] observed that platelet rich plasma contains more LPL activity than platelet poor plasma. The latter suggested that platelets may carry lipoytic activity absorbed on their surface. Soloff and Rutenberg found no effect on platelets on postheparin lipolytic activity. The discrepancy between the results of various authors may be related to the different experimental system used; the authors reporting an inhibition of LPL by platelets used an assay based on changes in optical density while Bucher used an assay based on the measurement of released free fatty acids. In our experiments we intend to compare both methods. Since platelets may absorb LPL as well as inhibit it partially, LPL activity will be assayed in four systems:
a) "post heparin" platelet poor plasma; b) "postheparin" platelet rich plasma; c) "post heparin" platelet poor plasma and normal platelets; d) normal platelet poor plasma and platelets obtained from "post heparin" subjects.

The effect of platelet extracts and the material released during platelet aggregation on the activity of LPL also will be investigated.

Clearance of postalimentary hyperlipaemia by heparin induced through activation of lipoprotein lipase, leads to an increase in the level of free fatty acids in serum. Whether this phenomenon may affect platelet function will be studied. Platelet aggregation in platelet rich plasma induced by ADP and other stimuli will be compared in four groups of individuals - a) fasting control; b) alimentary hyperlipaemia; c) fasting control injected with heparin; d) alimentary hyperlipaemia injected with heparin. Heparin is known to clear alimentary hyperlipaemia by release of fatty acids from chylomicrons and fatty acids are known to cause platelet aggregation[34]. Therefore it is possible that individuals on non restricted diets may have increased platelet aggregation after heparin injection.

Lecithin:cholesterol acyltransferase (LCAT) is the enzyme responsible for the conversion of free cholesterol to cholesterol ester in human plasma. It is our intention to examine whether cholesterol transferbetween platelets and plasma is promoted by the LCAT reaction, and to investigate the effects of platelets on the LCAT reaction.

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Cholesterol esterification will be measured by determining the amount of readioactive cholesterol that is esterified during the incubation period in the presence and absence of platelets. The labeled cholesterol will be added to plasma (which contains no active LCAT) as a serum albumin emulsion[31]. The reaction will be started by adding purified LCAT preparations to the radioactively labeled inactivated plasma. Esterification will be allowed to proceed to give linear rates over the selected time periods. Platelets will be isolated from human blood by the method of Mustard et al[26].

Interactions between Smoking and LCAT:

So called "risk factors" including smoking cannot be evaluated if genetic factors are ignored. Thus, homozygous familial hyperbetalipoproteinemic subjects have the highest incidence of coronary heart disease and at the youngest age whether they smoke or not. Statistical association can be misinterpreted as causal if there were still other as yet unknown genetic factors that make one more or less susceptible to coronary heart disease and/or if the genetic factors in some way predispose to smoking. Our ultimate aim is to determine whether LCAT activity which plays an important role in intravascular cholesterol metabolism is different in subjects with hypercholesteremia and in subjects with coronary heart disease than in healthy subjects and if so, whether such abnormalities are congenital or acquired and if they are influenced by smoking.

We have already shown that the initial fractional rate of serum cholesterol esterification is subnormal in most patients with myocardial infarction and in subjects with familial hyperbetalipoproteinemia. We now have similar findings in over 100 smoking and non smoking patients with coronary heart disease documented by coronary arteriography. Smoking appears to play no role either in the development of coronary heart disease or in LCAT activity judged by the coronary arteriographic studies. However, an interpretation of our statistical analysis is complicated by the fact that our patients had been on various drugs for their heart disease at the time they were catheterized.

We are therefore embarking on a study of the effects of commonly used drugs for heart disease (digitalis, anti-anginal, anti-thrombotic, and anti-arrhythmic and anti-hypertensive drugs) on LCAT activity.

Furthermore as stated previously we are comparing our results obtained with our technique with that obtainable by GLC.

Dr. Boden, with a grant from you will be carrying out a series of experiments on dogs to determine the effects of nicotine on pancreatic and gastric function with particular reference to secretin. He has agreed to permit us to use blood from his dogs to determine the effects of nicotine on LCAT activity and on blood lipoproteins and hopefully to determine the origin and degregation of LCAT by sampling blood across the liver from the portal vein from the systemic vein and from a systemic artery. LCAT activity will be measured both by our technique and by GLC. It is anticipated that a solution to the problem should be definitively obtained once we have developed a radio-immune assay technique.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

SPACE: The facilities available for this work include a fully equipped biochemical laboratory with space for at least four investigators and an office with two desks. A small separate room is presently being made available to house our gas liquid chromatograph and digital integrator accessory. A cold-room, shared by three other investigators is located in the "old Medical School" building which has recently been extensively renovated and re-equipped for research.

EQUIPMENT: Hewlett Packard 5711 A Gas Chromatograph
Hewlett Packard 7671 A Automatic Sampler for G.C.
Perkins Elmer Automatic Amino-Acid Analyzer (Fels Institute)
Beckman Model E AnalyticalUltracentrifuge (Fels Institute)
Beckman L3-50 Preparative Ultracentrifuge (Infectious Disease Section)
Beckman Model L Preparative Ultracentrifuge
Beckman LS-100 Scintillation Counter
Nuclear Chicago Mark II Scintillation Counter
Gilford 240 U.V. and Visible Spepetrophoto Meter
Beckman DU-2
LKB Ultrorac Fraction Collectors
Two Buchler Fractomette 200 Fraction Collectors
LKB Uvicord II Monitor with Recorder
Radiometer pH Meter 51
Radiometer pH Stat with TTT III Titrator
Photovolt Densicord Gel Scanner with Inteprator
Radiometer Conductivity Meter
Chromato Vue Ultraviolet Viewing Box
Buchi Evaporator
Techni-Lab Heat Block
Mettler Automatic Semi-Micro Balance
Mettler P1000 Automatic "rough" Balance
Buchler Evapo-Mix
Canalco Disc Gel Electrophoresis Apparatus
Beckman Microzone Electrophoresis with Duostat Power Supply
Eberbach Shaking Water Bath
Miscellaneous Equipment for Column Chromatography and Thin Layer Chromatography including three Peristaltic and one Piston Action Pump

I have been asked to supervise a Research Lipid Laboratory in the Department of Obstretics and Gynecology which opened on January 14, 1974. In addition a Pediatric Lipid Clinic has been started at St. Christophers Hospital which is Temple's Pediatric Hospital. This will give us an opportunity of studying children at all ages. This new laboratory is well equipped and has adequate space for a large office and two large laboratories. This laboratory will give me control of any lipid studies we wish to do in the newborn and his relatives.

11. Additional Facilities required:

Densitometer with integrator for thin layer chromatograms (Schoeffel) This instrument will permit precise quantification and also save considerable technician's time.

12. Biographical sketches of investigator(s) and other professional personnel (append):
See appendix's 1-6

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See appendix 1, page 2

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Soloff, Louis A.	30%	-----
Rutenberg, Harold L.	20%	-----
Lacko, Andras G.	60%	9,759
Raja, K.G.	100%	11,660
Nowotny, Alois H. Consultant	as needed	-----
David, Joshua S.K.	100%	-----
Technical		
Baldwin, F.	100%	11,762
Nickols, M.	100%	9,400
Jetter, F.	25%	2,800
Fringe Benefits @ 13%		5,900
Sub-Total for A		51,281
B. Consumable supplies (by major categories)		
Chemicals, Radiochemicals, glassware, vials, caps, service, plasma, volunteers, etc.		5,000
Sub-Total for B		5,000
C. Other expenses (itemize)		
Travel		500
Secretarial Services		500
Sub-Total for C		1,000
Running Total of A + B + C		57,281
D. Permanent equipment (itemize)		
Densitometer with integrator for thin layer chromatograms (Schoeffel)		10,000
Sub-Total for D		10,000
E. Indirect costs (15% of A+B+C)		E 8,592
		Total request $ 75,873

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	53,845	5,250	1,050	------	9,022	$69,167
Year 3	56,537	5,513	1,100	------	9,473	$72,623

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Physiological Studies on LCAT	Council for Tobacco Res. U.S.A.	62,500	1973-74
Purification and Chemical Characterization of Human Plasma LCAT	NIH	92,673	1973-76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
Temple University Health Sciences Center
% Mr. David W. Siegel

Mailing address for checks
3400 North Broad Street
Phila. Penna. 19140

Principal investigator
Typed Name Louis A. Soloff, M.D.
Signature [signature] Date 1/21/74
Telephone 215 (Area Code) 221 (Number) - 3343 (Extension)

Responsible officer of institution
Typed Name Samuel S. Herman, Ph.D.
Title Associate Vice President for Research
Signature [signature]
Telephone 215 (Area Code) 221-3250 (Number) (Extension)

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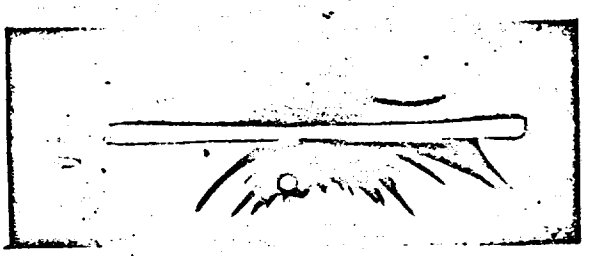
Fig. 1



Preliminary additional work suggests that this arc is the enzyme and not enzyme contaminated with HDL or albumin.

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